SUPPLEMENTAL DATA
Suzuki-Anekoji *et al.*

EXPERIMENTAL PROCEDURES

Cells and antibodies-
C6 cells, U87-MG cells and an HNK-1 hybridoma were purchased from American Type Culture Collection. HNK-1 antibody was purified from hybridoma culture supernatants using an IgM purification kit (Pierce Chemicals). Antibodies were purchased as follows: Antibodies against laminin-binding glycan of α-dystroglycan (IIH6C4, Millipore), β1-integrin (mAb13, human CD29, BD Biosciences), α6-integrin (GoH3, CD49f, BD Biosciences), total ERK1/2 (rabbit polyclonal) and phospho-ERK 1/2 (D13.14E, p42/44 MAPK, Cell Signaling Technologies), total FAK (focal adhesion kinase, rabbit polyclonal, BD Biosciences), Y^{397} phospho-FAK (141-9, Invitrogen), vimentin (V9, DAKO Cytomation), NCAM (rabbit polyclonal, Chemicon), laminin1+2 (rabbit polyclonal, Gene Tex), Iba-1 (rabbit polyclonal, Wako Pure Chemicals) and HRP-conjugated rat anti-mouse IgM (LO-MM-9, Invitrogen). Laminin peptide E3 and anti-α-dystroglycan core peptide antibody DG203 were prepared as described previously (*1*).

HNK-1 overexpression in C6 glioma cells-
Rat astrocytoma C6 cells endogenously express HNK-1 sulfotransferase, but not GlcAT-P. To create HNK-1 glycan positive C6 cells, we transfected rat C6 glioma cells with GlcAT-P (*2*) using LipofectAmine PLUS. Hygromycin B-resistant clones were screened by immunocytochemistry and flow cytometry. Initially, clone G14 was selected. Control C6 cells were obtained by mock transfection of pcDNA3.1 (+)-hyg (Invitrogen). At a second screening, control C6 cells were further co-transfected with pcDNA3.1-zeo and pcDNA3-GlcAT-P. Zeocin-resistant HNK-1-positive clones were selected by immunocytochemistry and designated clones H1-H7. In this study, mock-transfected C6 cells, G14 and H1 clones were used. HNK-1 expression was confirmed by flow cytometry (FACSCalibur, BD Biosciences).
Similarly, HNK-1 positive stable cell lines of human glioma U87 cells were generated by transfection of rat GlcAT-P, named clone positive-1 to -10. Mock transfectant was also generated with pcDNA3.1(+)-hyg plasmid, screened with Hygromycin B resistance. Expression of HNK-1 was confirmed by immunocytochemistry. Growth curve of mock clone no.8, and HNK-1 positive clones pos-2 and pos-10 are shown in Fig. S7.

RT-PCR-
Total RNA was extracted from cultured cells with Trizol reagent (Invitrogen) and treated with DNase. cDNA was transcribed using SuperScript II reverse transcriptase (Invitrogen) together with an oligo $(dT)_{12-18}$ primer. The sequences of primers used for PCR were: rat (common sequence to mouse) HNK-1 ST, 678-1000 (323bp), 5'-GAAGAACCGGACAGAGACCC-3' (5'-primer); 5'- GCCGGATGTCTCGTTTGCTG-3' (3'-primer); rat GlcAT-P, 550-757 (207 bp), 5'-AATCTGGCCCTGCGCTGGTT-3' (5'-primer); 5'-CAACCACTTTCCCTGCCCCA-3' (3'-primer). Control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were described previously (*3*). PCR was carried out at 95°C for 5 min, followed by 35 cycles of 95°C for 1min, 57°C for 1min, and 72°C for 1min, and by a final incubation at 72°C for 8 min.

Immunoblot analysis and laminin overlay -
Cell lysates were prepared in RIPA solution (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) containing Complete protease inhibitor cocktail (Roche) and phosphatase inhibitors (1mM sodium orthovanadate and 10mM sodium fluoride). Cells were lysed by passage through a 28G needle several times, sonicated in iced water for 15 min, then the supernatant from a 12,000xg centrifugation was used as a total cell lysate. Protein concentration was determined using a micro BCA kit (Pierce).

Monodispersed cells were attached to laminin-coated plates to stimulate phosphorylation of kinases, and cell lysates were prepared at times indicated. Cell lysates were subjected to SDS-PAGE and immunoblotting with anti-phosphorylated ERK or phosphorylated FAK. Blots were then stripped for blotting for total ERK1/2 or FAK.

For immunoprecipitation, 0.5 to 1 mg of total protein was precleared with anti-mouse IgM beads and mixed with 30 μg of HNK-1 antibody, followed by 45 μl of anti-mouse IgM beads overnight. Beads were washed with RIPA buffer and PBS, and the remaining antibody complex was subjected to SDS-PAGE and immunoblotting. Bands were visualized using an ECL PLUS kit (Amersham/GE Health Sciences).

A laminin overlay was performed as described (*1*) using ligand-binding buffer (LBB;10mM triethanolamine-acetate pH7.6, 140mM NaCl, 1mM $MgCl_2$, and 1mM $CaCl_2$) instead of TBS-T and anti-laminin antibody.

Cell binding assay-

Ninety six-well plates were coated with the three-times serial dilution of laminin (Lam-111), fibronectin, or laminin E3 peptide solution and incubated overnight at 4 ºC. Wells were blocked with 2.5% BSA (bovine serum albumin) in PBS for one hour. Wells were washed with LBB, $2x10^5$ cells per well were applied in LBB, and plates were incubated at 37ºC for 30 minutes. Cells were washed with 200μl of LBB four times with 5 minutes of rotation, and remaining cells were fixed and photographed under an inverted microscope. Bound cell numbers were counted manually, showing an average of four fields per well. Duplicate wells are shown in the binding curve.

Microglia cytotoxicity assay-

Cytotoxicity of the rat microglial cell BV2 against C6 cells was measured using a ToxiLight® BioAssay Kit (Lonza Rockland, Inc., ME), following the manufacturer's protocol (*4, 5*). Since cell size differs between C6 and G14 lines, 2.4×10^4 of C6 cells or 1.0×10^4 of G14 cells were plated per well in a 96-well microtiter plate, to provide the same intensity of luminescence. On top of the target (T) cells, BV2 (rat microglial) effector (E) cells, a kind gift of Dr. Yunwu Zhang (Sanford-Burnham Medical Research Institute), were plated at an E:T ratio of 1:1 to 32:1. Microglial cells were stimulated using two stimulants, 1 μg of lipopolysaccharide (LPS) or 0.1 μg of interferon-γ (IFNγ) in 100 μl of 10% FBS-α-MEM media per well, followed by incubation at 37ºC in 5% CO_2. Nine, 24 and 46 hours after induction, a 20 μl aliquot of the culture supernatant was transferred to a new plate and mixed with 100 μl of detection solution, and the amount of adenylate kinase released from lysed target cells was measured using a luminometer. Data collected 46 hours after the stimulation is shown in Figure S2.

REFERENCES

1. Bao, X. , Kobasyashi, M. , Hatakeyama, S. , Angata, K. , Gullberg, D. , Nakayama, J. , Fukuda, M-N. , and Fukuda M. (2009) *Proc. Natl. Acad. Sci.* **106**,12109-12114
2. Ong, E. , Yeh, J-C. , Ding, Y. , Hindsgaul, O. , and Fukuda, M. (1998) *J. Biol. Chem.* **273**, 5190-5195
3. Angata, K. , Huckaby, V. , Ranscht, B. , Terskikh, A. , Marth, J-D. , and Fukuda, M. (2007) *Mol. Cell Biol.* **27**, 6659-6668
4. Blasi, E. , Barluzzi, R. , Bocchini, V. , Mazzolla, R. , and Bistoni, F. (1990) *J. NeuroImmunol.* **27**, 229-237
5. Crouch, S-P-M. , Kozlowski, R. , Slater, K-J, and Fletcher, J. (1993) *J. Immunol. Methods* **160**, 81-88

SUPPLEMENTAL FIGURES

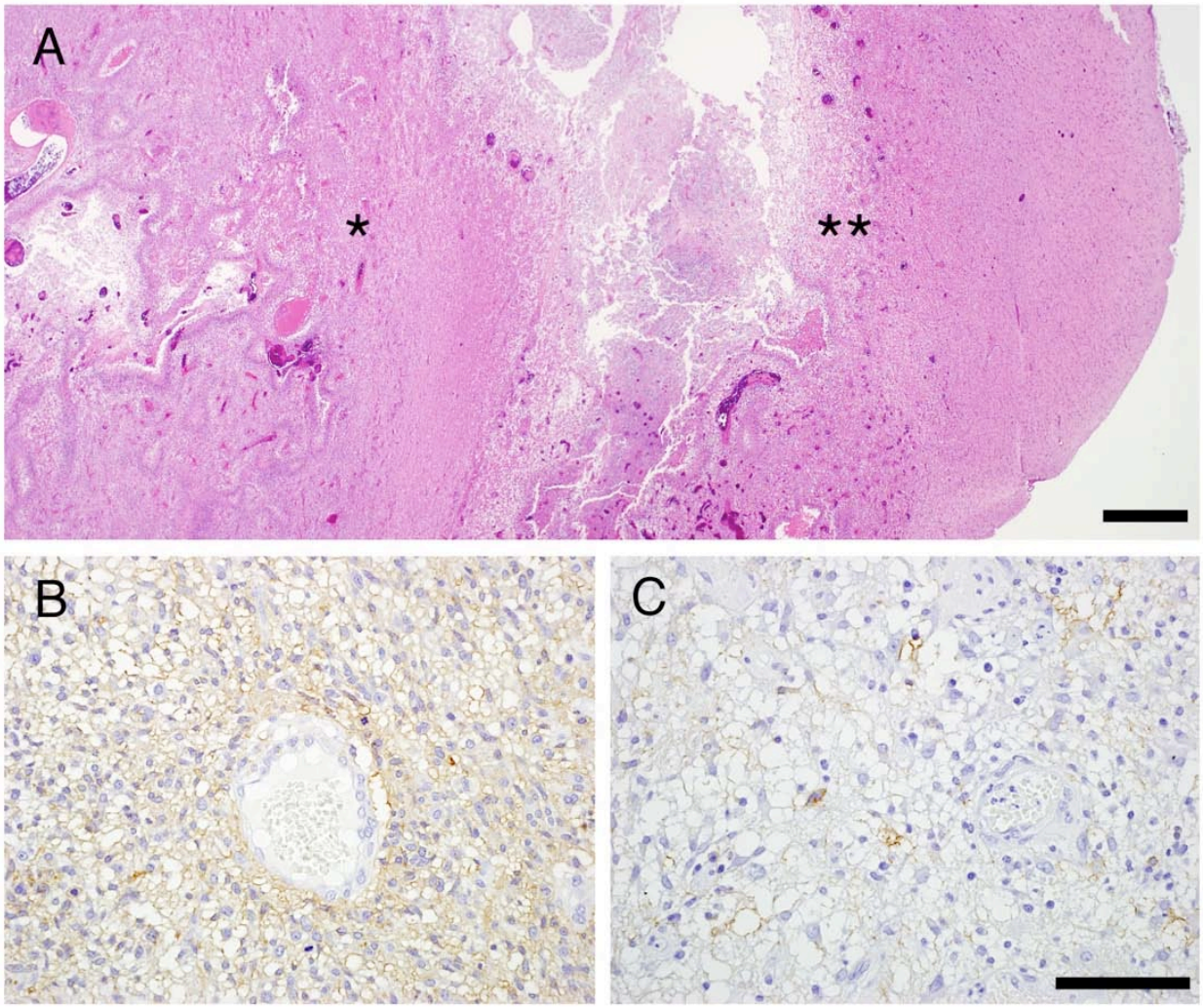

Fig. S1: Differential expression of HNK-1 glycan in a patient with glioblastoma. *A*, Histology of a glioblastoma. Note that pseudopalisading necrosis is prominent. *B*, Expression of HNK-1 glycan in the central portion of the tumor (* in *A*). *C*, Expression of HNK-1 glycan in the invasive front of the tumor (** in *A*). Bars in *A* and *C* are 1 mm and 100 μm, respectively.

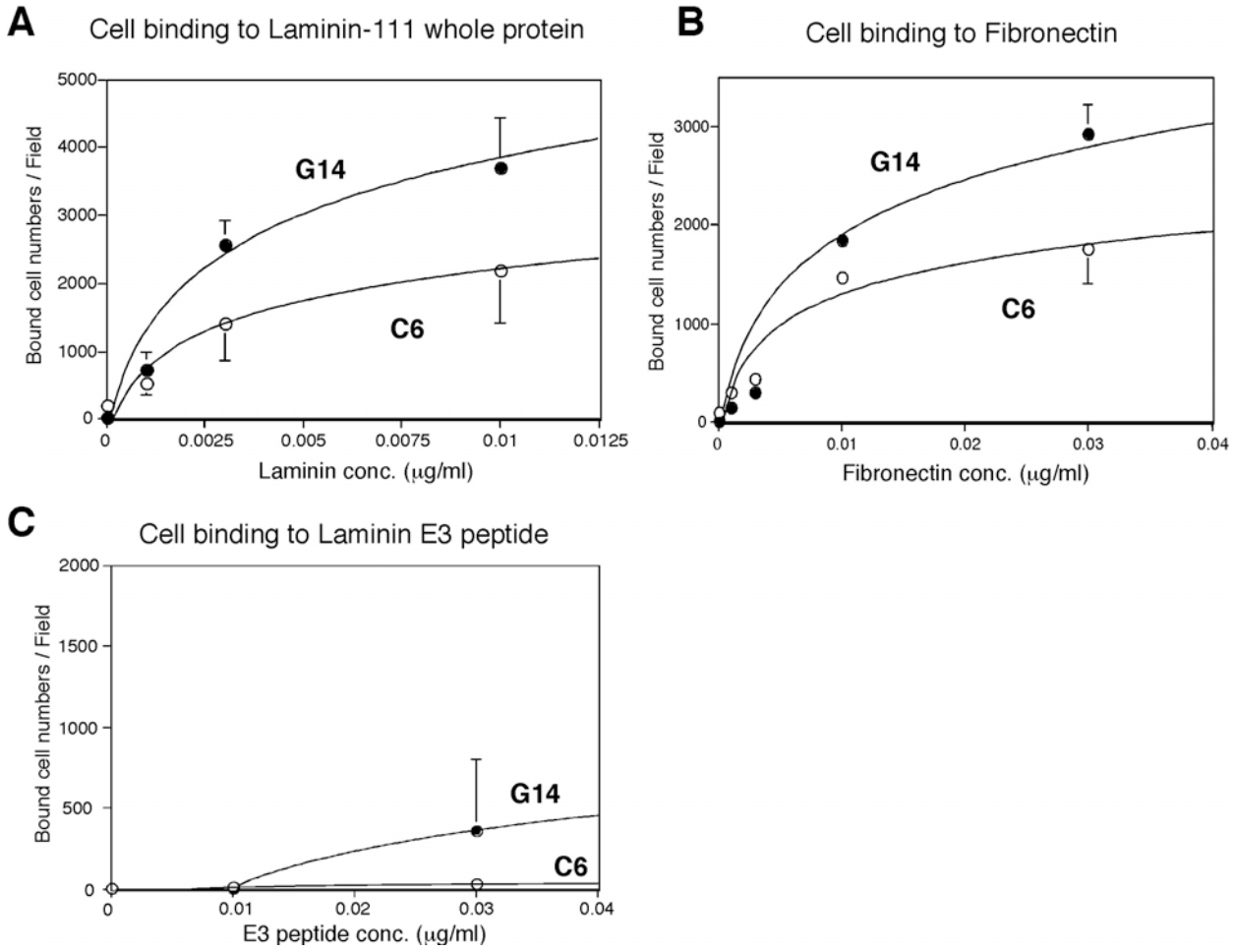

Fig. S2: Binding of control C6 or C6-HNK-1 (G14) cells was tested on matrix-coated 96-well-plates. ***A***, Cell binding to laminin whole protein. ***B***, Cell binding to fibronectin. ***C***, Binding to laminin-E3 peptides. Note that scale differs in *C*. Affinity of cells is greatest to laminin > fibronectin > laminin E3 peptide, but in all cases, G14 cells bound more efficiently than did C6 cells.

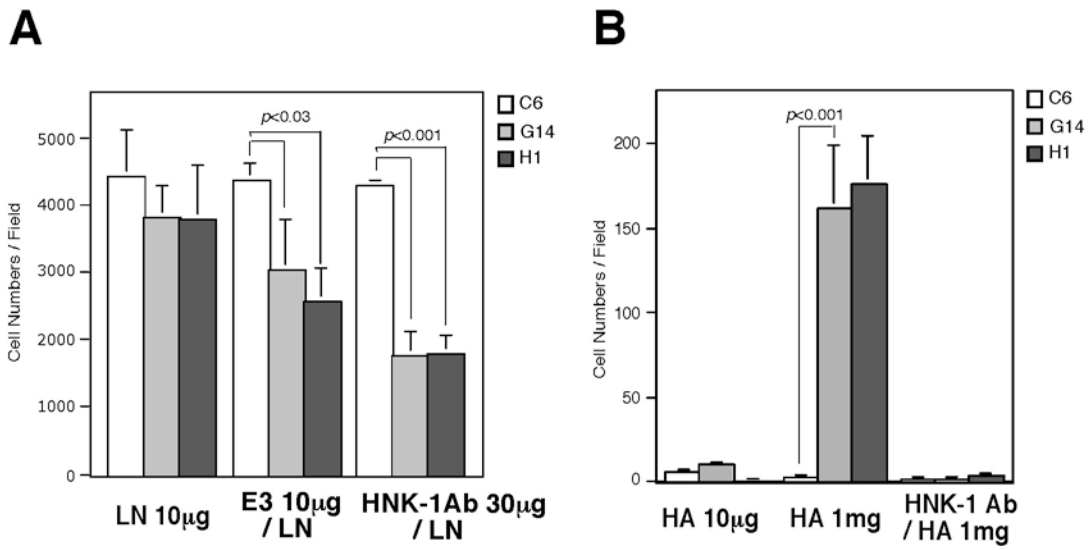

Fig. S3: Cell migration assay of C6, G14 and H1 cells in the laminin (LN) coated or hyaluronic acid (HA) coated transwell chamber. ***A*,** The bottom surface of the transwell was coated with 10 μg/ml of laminin-111, cells were applied in the upper chamber pre-mixed with 10 μg/ml of E3 peptide (E3 10μg/LN), 30 μg/ml of HNK-1 antibody (HNK-1Ab 30μg/LN) or without treatment (LN 10μg). Both of G14 and H1 cells migrations were attenuated by addition of either E3 peptide or HNK-1 antibody, but not as much as it inhibited in the fibronectin (Fig. 4-B). ***B***, Hyaluronic acid (HA) was coated at the bottom surface of transwell chamber in the concentration of 10 μg/ml or 1mg/ml, then migration of C6, G14 and H1 cells were observed. Although cells did not migrate in the 10 μg/ml of HA, notably, HNK-1 positive cells (G14 and H1) migrated in the 1mg/ml of HA. This migration was inhibited by addition of 30 μg/ml of HNK-1 antibody in the upper chamber (HNK-1 Ab/HA 1mg). The experiments were carried out for three times in A and B.

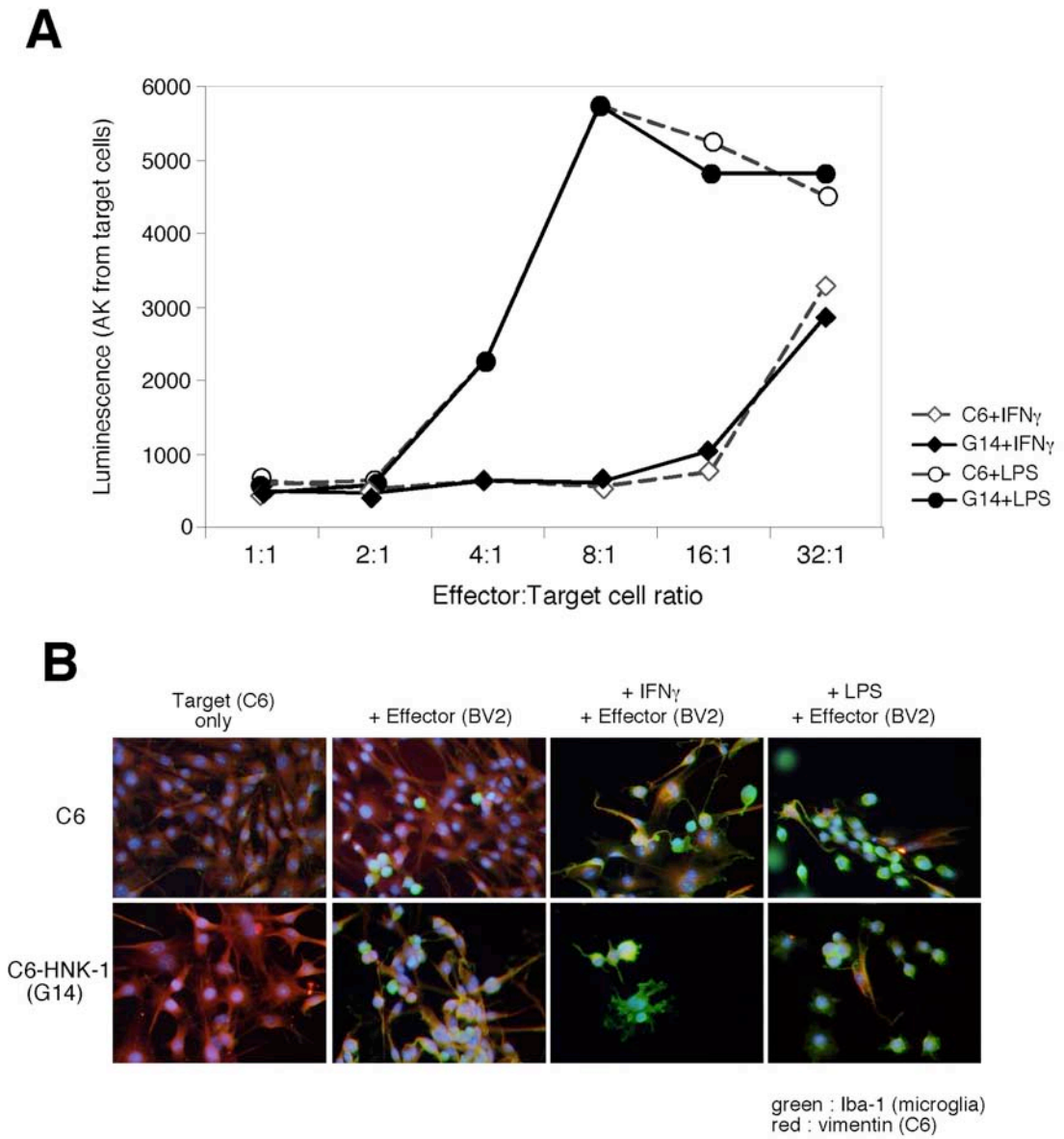

Fig. S4: The cytotoxic effect of microglial BV2 cells on C6 cells. ***A***, C6 or G14 cells are plated, microglial BV2 cells were added, then cyotolytic reaction was stimulated by addition of either LPS or interferon-γ. Adenylate kinase (AK) activity released from lysed target cells (C6, open circles or G14 cells, closed circles) was detected as luminescence. ***B***, Images of control (C6) and C6-HNK-1 cells (G14) before and after stimulation of cytolysis. C6 or G14 cells (red) were detected by anti-vimentin and Rhodamine-conjugated anti-mouse IgG antibodies, while microglial cells were detected by anti-Iba-1 and Alexa Fluor 488 conjugated anti-rabbit IgG antibodies.

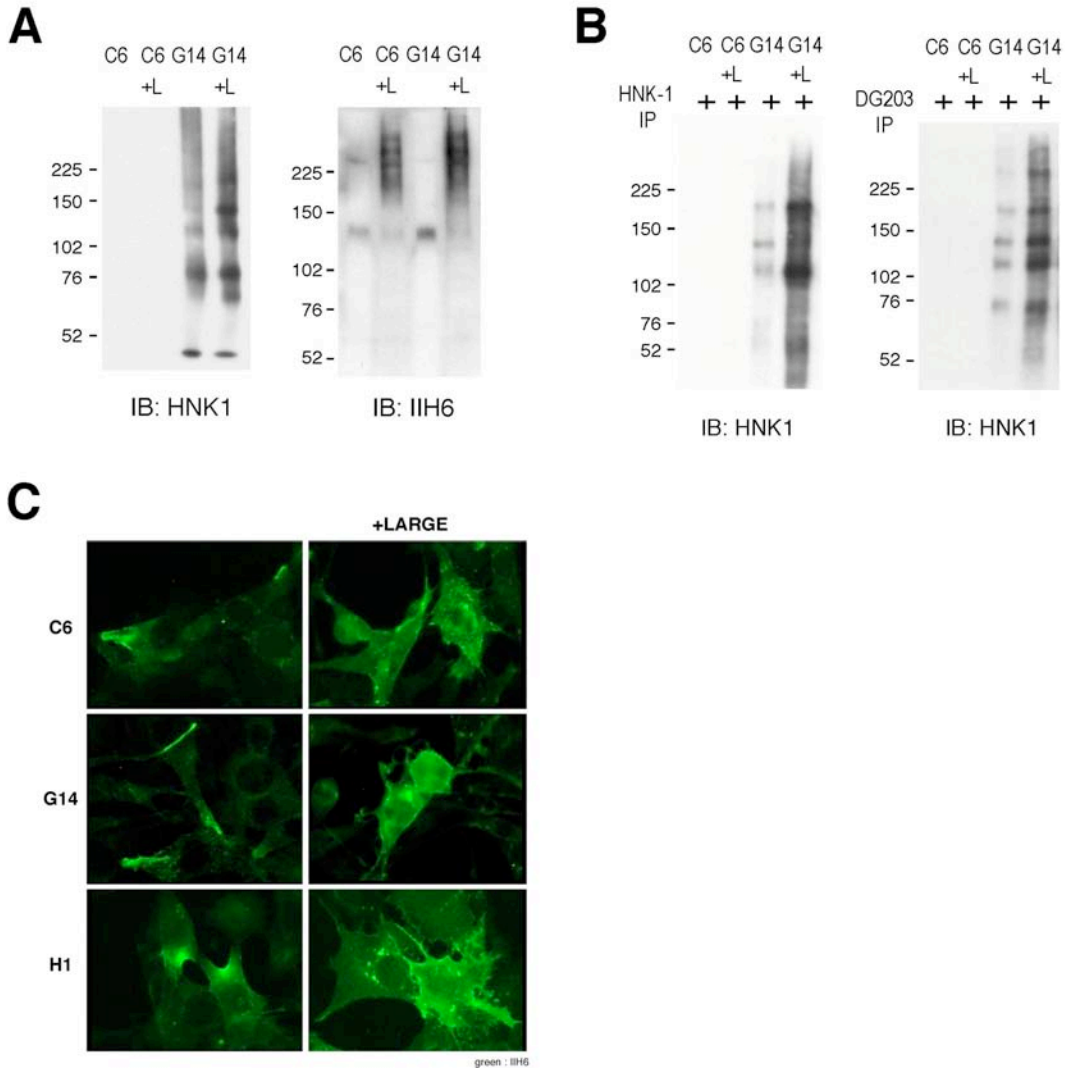

Fig. S5: Endogenous expression of α-dystroglycan in C6 cells. ***A***, Immunoblot of total cell lysate, with or without LARGE transient transfection (L). IIH6 recognizes laminin-binding glycan of α-dystroglycan. ***B***, Cell lysates from (A) were immunoprecipitated by either HNK-1 antibody or DG203 α-dystroglycan core peptide antibody, then subjected to immunoblot with HNK-1. Panels (A) and (B) suggest that endogenous α-dystroglycan in G14 cells is modified by both of HNK-1 and laminin-binding glycans. ***C***, C6, G14 and H1 cells were transiently transfected with LARGE, stained by IIH6 and FITC anti-mouse IgM antibodies to detect laminin-binding glycans. Without LARGE transfection, cells express moderate amount of IIH6 epitope regardless of HNK-1. With LARGE, expression of IIH6 epitope is enhanced overall on cell surface.

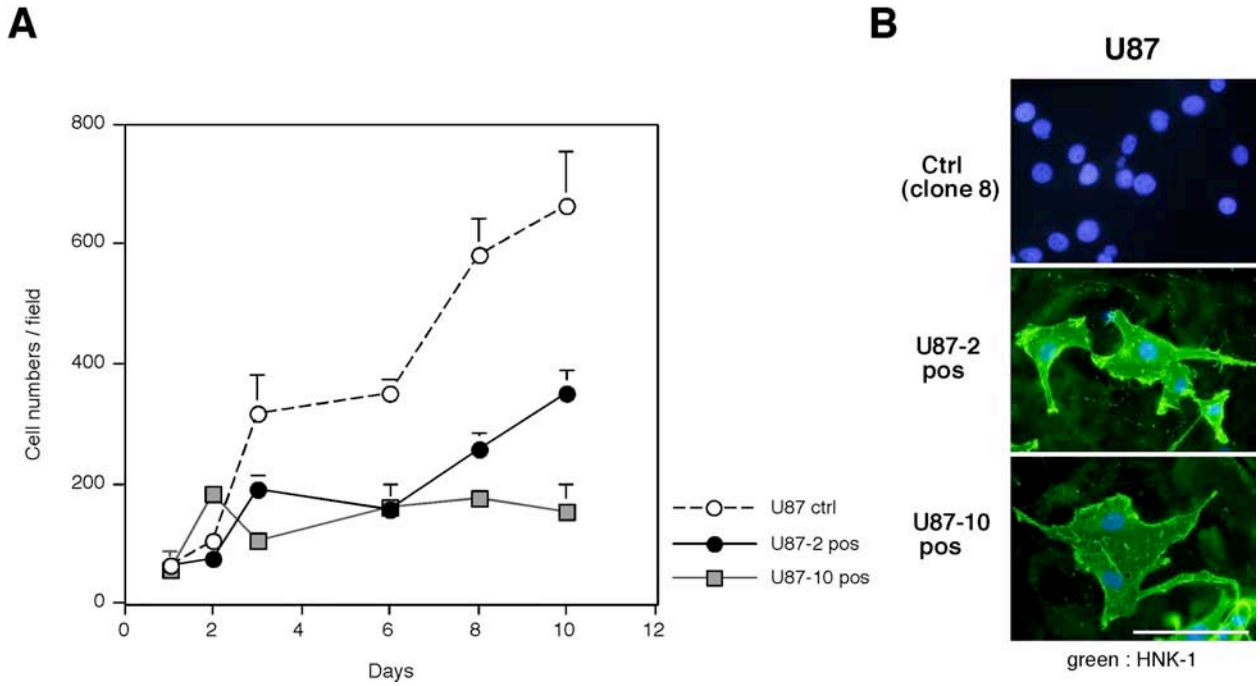

Fig. S6 Cell proliferation rate of HNK-1 positive U87 human glioma cells.
(A), Growth curve of HNK-1 negative (ctrl) and HNK-1 positive (U87-2 pos and U87-10 pos) U87 cells. (B) HNK-1 staining of clones. HNK-1 positive U87 stable cell lines are generated by transfection of rat glucuronyltransferase-P, as described in Experimental Procedures.